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The Bacterial Oxidation of Nicotine

III. THE ISOLATION AND IDENTIFICATION OF 6-HYDROXYPSEUDOOXYNICOTINE*

LAWRENCE I. HOCHSTEIN† AND SYDNEY C. RITTENBERG

From the Department of Bacteriology, University of Southern California, Los Angeles, California

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Previous studies in this series (1, 2) have established that an enzyme fraction, obtained from the nicotine-oxidizing bacterium P-34, catalyses the oxidation of nicotine and 6-hydroxynicotine with the consumption of 1 μmole and 0.5 μmole of oxygen, respectively, per μmole of substrate. This paper reports on the isolation of the product of nicotine and 6-hydroxynicotine oxidation by the above mentioned enzyme fraction and the identification of the isolated compound as 6-hydroxypseudooxynicotine.

EXPERIMENTAL

The preparation of cell-free extracts, ultraviolet spectrophotometry, paper chromatography, optical rotation, and melting point determinations as well as manometric determinations of oxygen consumption and carbon dioxide release were performed as previously described (1, 2). Ammonia was collected by the Conway microdiffusion technique (3), and determined by direct nesslerization of the trapped ammonia.

6-OHN[1] was isolated from growth medium as the silicotungstic acid salt and purified by a modification of the previously described method (2). A solution of the free base, regenerated from its silicotungstic acid salt (4), was acidified with hydrochloric acid to pH 2, and placed on a Dowex 50 column in the hydrogen form (1.5 × 21 cm). The column was washed with water until the effluent exhibited a low and constant absorbancy at 232 and 295 mμ. A gradient elution schedule was then initiated employing 3 M ammonium hydroxide in the reservoir and 2.5 liters of distilled water in the mixing chamber. Fractions were collected in 18-ml lots; after 216 ml of effluent had been collected, 6-OHN, as judged by the increase in absorbancy at 232 and 295 mμ, appeared in the following 108 ml. The fractions containing 6-OHN were pooled, acidified to pH 2 with hydrochloric acid, and evaporated to dryness. 6-OHN was extracted from the dry residues with boiling Skellysolve B and crystallized from the same solvent.

RESULTS

Oxidation of 6-OHN—The 40-60 fraction,[2] which catalyzed the aerobic oxidation of nicotine only in the presence of methylene blue (1), oxidized 6-OHN in the absence of the dye. Oxidation ceased after the consumption of 0.5 μmole of oxygen per μmole of 6-OHN. No production of carbon dioxide or of significant quantities of ammonia was observed (Table I).

The rate of oxidation exhibited a marked dependence upon pH. When determined in Tris-maleate buffer, the optimum occurred at pH 8 (Fig. 1). 6-OHN oxidation was a function of the enzyme concentration up to 1.05 mg protein per ml of reaction mixture (Fig. 2). When the enzyme concentration was at excess (3.7 mg protein per ml of reaction mixture), the rate of 6-OHN oxidation was independent of the substrate concentration over the range tested (7×10^{-4} M to 1.05×10^{-2} M).

At the termination of 6-OHN oxidation, reaction mixtures contained a substance having an absorption maximum at 289 mμ when determined in 0.1 N hydrochloric acid. When the spectra were determined in 0.1 N sodium hydroxide, the maximum exhibited a reversible bathochromic shift to 310 mμ. Identical absorption characteristics were exhibited by reaction mixtures obtained by incubating nicotine with crude enzyme and methylene blue and stopping the oxidation when 1 μmole of oxygen per μmole of nicotine had been consumed. Three volumes of 0.1 N HCl were added to samples of both types of reaction mixtures and the precipitated protein was removed by centrifugation. Portions of the supernatant were spotted on Whatman No. 1 paper and chromatographed with butanol-benzene-0.2 M sodium acetate buffer, pH 5.6 (85:5:30) as the solvent and Dragendorff's reagent as the indicator. Both types of reaction mixtures contained a compound of R_F 0.09, which was not evident in mixtures devoid of substrate. In the solvent system used, nicotine and 6-OHN had R_F values of 0.32 and 0.15, respectively. These data indicate that the compounds formed by the crude extract, at the expense of nicotine, and by the 40-60 fraction, at the expense of 6-OHN, were identical.

Enzymatic Synthesis and Isolation of Product—In a typical experiment, the following components were added to each of two 250-ml Erlenmeyer flasks: 1250 μmoles of 6-OHN, previously adjusted to pH 7.9 with hydrochloric acid; 2 ml of a 40-60 fraction (116 mg of protein); and water to a total volume of 20 ml. The flasks were shaken in a 30° water bath. The reaction was followed manometrically by simultaneously incubating 2 ml of the above reaction mixture in a Warburg flask. When oxidation ceased, the reaction mixtures were pooled, brought to 70° for 5 minutes, and the denatured protein was removed by centrifugation.[3] The clear supernatant was lyophilized and the residue

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† Present address, Biochemical Research Laboratory, Elgin State Hospital, Elgin, Illinois.

[1] The abbreviations used are: 6-HPO, 6-hydroxypseudooxynicotine; 6-OHN, 6-hydroxynicotine.

[2] That portion of the crude extract which precipitated at 40 to 60 per cent saturation with respect to ammonium sulfate is designated as the 40-60 fraction.

[3] Failure to immediately denature the enzyme at this stage results in the formation of a blue pigment. This pigment formation in nicotine degradation will be discussed in a subsequent publication.

TABLE I

Oxidation of 6-OHN by 40-60 Enzyme Fraction

The complete system contained the following in a total volume of 2.0 ml: 14 μmoles of 6-OHN; 1.25 μmoles of methylene blue (MB); 10 μmoles of potassium phosphate buffer, pH 7; 2.1 mg of 40-60 fraction. Gas phase air; 30°. The 40-60 fraction was dialyzed overnight against distilled water.

Condition	μMoles per μmole of 6-OHN		
	Oxygen uptake	CO_2 formed	NH_3 formed
Complete system	0.52	0	
Minus 6-OHN	0	0	
Minus MB	0.46	0	0.20
Minus MB and 6-OHN	0	0	0.18

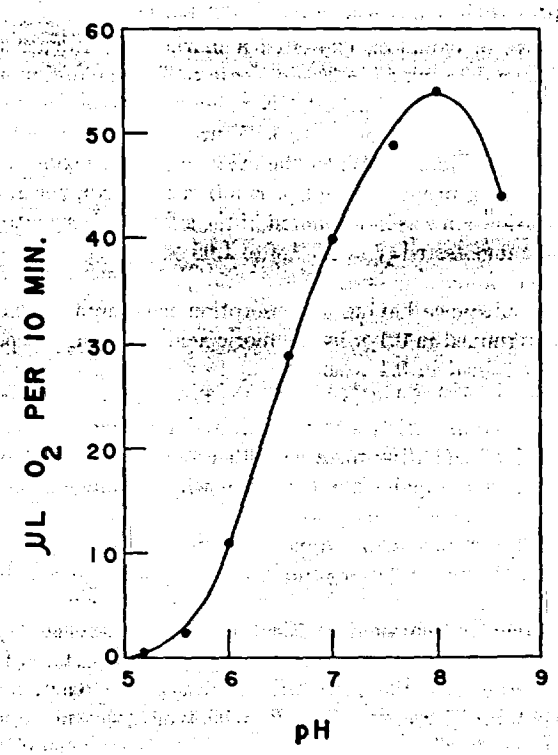

FIG. 1. The effect of pH on the oxidation of 6-OHN. The reaction mixtures contained the following in a total volume of 2.0 ml: 6-OHN, 14 μmoles; Tris-maleate buffer, 100 μmoles, at the desired pH; 40-60 fraction, 1.4 mg protein. Gas phase air, 30°.

extracted with 50 ml of absolute ethanol previously acidified with dry hydrochloric acid. The alcoholic extract was warmed and anhydrous ethyl ether was slowly added with constant shaking until a permanent faint turbidity was obtained. The solution then was cooled to room temperature and placed at −10° for at least 12 hours. The resulting precipitate was filtered, washed with cold (−10°) absolute ethanol, and dried in a vacuum. The washings and the filtrate were combined and the ether precipitation repeated through several cycles until further treatment failed to yield a precipitate (total yield of material was 695 mg). The initial precipitates in a series were usually somewhat resinous and dark colored. Succeeding fractions became lighter in color and amorphous, or even crystalline, if the ether additions were carefully controlled, and, in 0.1 HCl, they showed a progressive increase in absorption at 289 mμ. A small portion, 75 mg, of

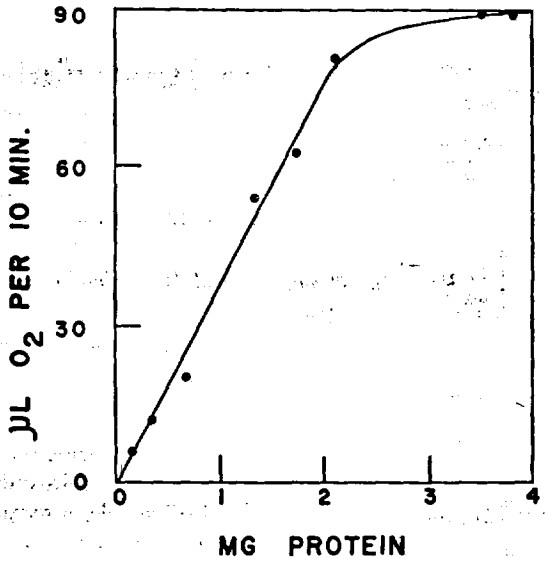

FIG. 2. The effect of enzyme concentration on the oxidation of 6-OHN. The reaction mixtures contained the following in a total volume of 2.0 ml: 6-OHN, 14 μmoles; Tris-maleate buffer, pH 8.0, 100 μmoles; and 40-60 fraction protein as indicated. Gas phase air, 30°.

a nonresinous fraction of high absorption was redissolved in acid ethanol, decolorized with Norit A, and reprecipitated with ethyl ether as above. The final product, 46 mg, consisted of white crystalline needles that melted at 157–158° (uncorrected).

The isolated product had a single absorption maximum whose position was pH dependent (Fig. 3). At a pH of less than 8 the peak occurred at 289 mμ (ϵ = 16,000), at a pH between 8 and 12 at 328 mμ (ϵ = 22,000), and at a pH greater than 12 at 310 mμ (ϵ = 21,000).

The absorption spectrum of the isolated product suggested the presence of a new double bond in conjugation with the pyridine ring (5). This, coupled with the stoichiometry of oxygen consumption and the failure to detect optical activity in the product further indicated that oxidation resulted in the destruction of the molecule's center of asymmetry located at carbon 5 of the pyrrolidine ring. Dehydrogenation at this position would yield 6-hydroxy-*N*-methylmyosmine; however elemental analysis[4] indicated than an hydrolytic as well as an oxidative step had occurred and suggested that the product isolated was a dihydrochloride of 6-HPO:

$C_{10}H_{14}N_2O_2 \cdot 2HCl$

Calculated: C 44.94, H 5.99, N 10.49, Cl 26.59

Found: C 45.44, H 5.95, N 10.55, Cl 27.27

The identity of the isolated product was established as 6-HPO by comparing it to the product of the enzymatic hydroxylation of pseudooxynicotine.[5] The enzyme fraction employed has been shown to hydroxylate a number of compounds related to nicotine, including pseudooxynicotine, at the 6-position of the pyridine ring (6). It was found that following hydroxylation of pseudooxynicotine, the original absorption maxima located at 223 mμ and 264 mμ were replaced by a single maximum

[4] The analysis was performed by Dr. A. Elek, Elek Micro Analytical Laboratories, Los Angeles, California.

[5] Kindly supplied by Dr. C. H. Rayburn.

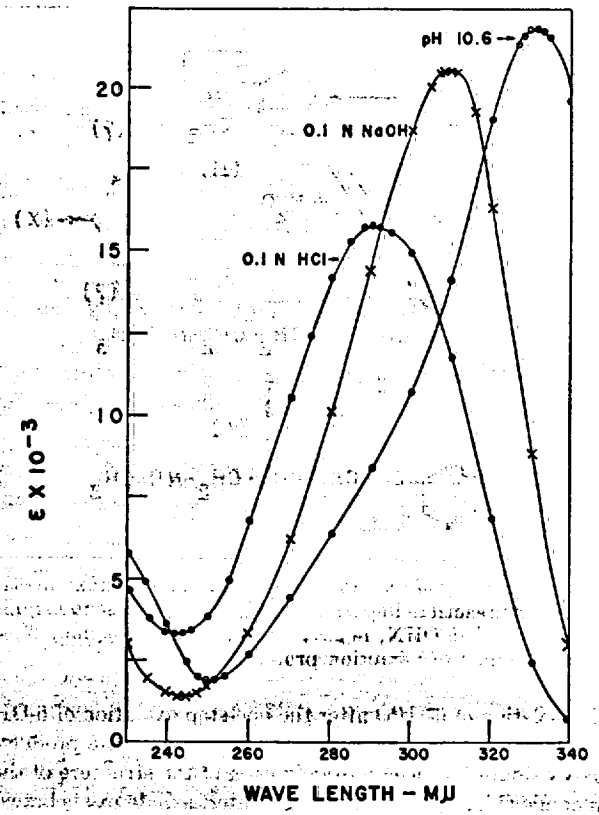

FIG. 3. The absorption spectrum of the isolated product. 0.1 N HCl, ●——●; pH 10.6, ○——○; 0.1 N NaOH, ×——×.

whose location depended upon pH in a manner identical to the behavior of the product of 6-OHN oxidation. In addition, the chromatographic characteristics of the products from both substrates were identical (Table II).

Metabolism of 6-HPO—Nicotine-grown resting cells oxidized 6-HPO at approximately the same rate as they oxidized nicotine. At the cessation of oxidation, oxygen consumption was 6.3, 5.7, and 5.3 μmoles of oxygen per μmole of nicotine, 6-OHN, and 6-HPO, respectively (Fig. 4).

Crude cell extracts, which oxidized nicotine with the consumption of 2.1 μmoles of oxygen per μmole of nicotine, oxidized 6-HPO with the consumption of 1 μmole of oxygen per μmole of substrate. The rate of oxidation of 6-HPO and nicotine were essentially identical after oxidation of the latter had proceeded beyond 0.5 μmole of oxygen per μmole of nicotine (Fig. 5).

When 6-HPO was incubated with an unsupplemented 40-60 fraction, no oxygen consumption was observed but a blue pigment apparently identical to that found in the growth medium (7) was observed. If the 40-60 fraction was supplemented with brilliant cresyl blue, oxidation of 6-HPO occurred with the consumption of 0.5 μmole of oxygen per μmole of substrate. In the presence of methylene blue,[6] the 40-60 fraction oxidized 6-HPO to various oxidation states; oxidations consuming as little as 0.5 μmole and as much as 1.5 μmoles of oxygen per μmole of 6-HPO have been observed with different preparations.

After oxidation of 6-HPO by the 40-60 fraction in the presence

[6] Contrary to what had previously been observed (1) the 40-60 fraction prepared from most batches of cells will oxidize nicotine beyond the oxidation state of 6-HPO (1 μmole of oxygen per μmole of nicotine) in the presence of methylene blue.

TABLE II

Comparison of products derived from 6-OHN oxidation and pseudooxynicotine hydroxylation

The hydroxylation of pseudooxynicotine was carried out in the following manner: 10 μmoles of pseudooxynicotine, 0.125 μmole of methylene blue, 10 μmoles of potassium phosphate buffer, pH 7.0, and the "hydroxylating enzyme" were incubated in a total volume of 2.0 ml at 30° until oxidation ceased (0.45 μmole oxygen per μmole of pseudooxynicotine). Aliquots of the reaction mixture were adjusted to the appropriate pH with HCl or NaOH as required and the absorption spectrum was determined. For chromatography, the acidified reaction mixture was spotted on Whatman No. 1 paper and developed in butanol-ethanol-water (42:42:16). The spots were visualized by spraying the paper with Dragendorff's reagent. Under these conditions, pseudooxynicotine has an R_F of 0.96.

Product source	Absorption maximum			E_M		R_F
	In 0.1 N HCl	At pH 10.6	In 0.1 N NaOH	310/289	328/310	
	mμ	mμ	mμ			
6-OHN	289	328	310	1.16	1.10	0.91
Pseudooxynicotine	289	328	310	1.17	1.09	0.91

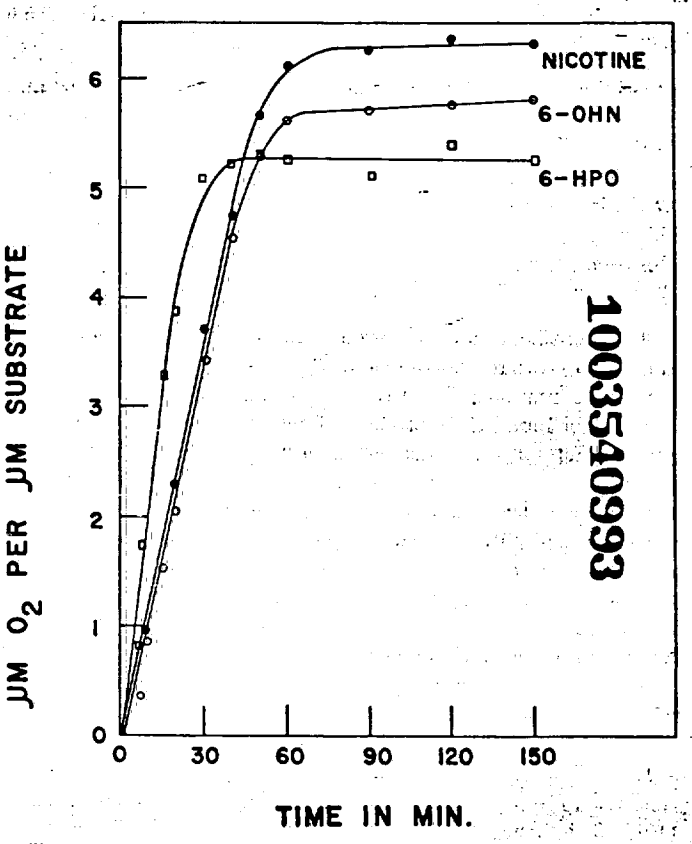

FIG. 4. The oxidation of nicotine, 6-OHN, and 6-HPO by nicotine-grown resting cells. The reaction mixtures contained the following in a total volume of 2.0 ml: potassium phosphate buffer, pH 7, 70 μmoles; 0.25 ml of an 18-hour culture of nicotine-yeast extract grown cells (strain P-34) equivalent to 731 Klett turbidity units (No. 60 filter); and either nicotine, 4 μmoles; 6-OHN, 4.5 μmoles; or 6-HPO, 1.9 μmoles as noted. Gas phase air, 30°. Data corrected for autorespiration.

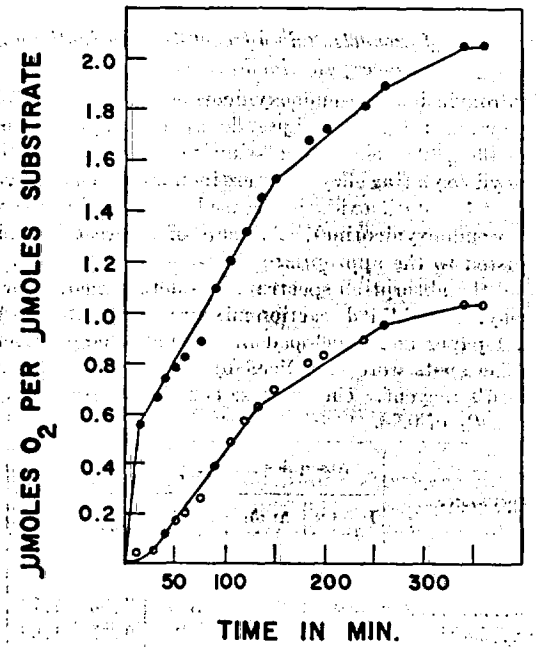

FIG. 5. The oxidation of 6-HPO and nicotine by crude cell extracts. The reaction mixtures contained the following in a total volume of 2.0 ml: potassium phosphate buffer, pH 7.0, 100 μmoles; methylene blue, 0.625 μmole; crude enzyme, 5 mg protein; and where indicated, nicotine, 4 μmoles, ●——●; or 6-HPO, 5.6 μmoles, ○——○. Gas phase air, 30°.

of brilliant cresyl blue, the reaction mixture showed a new and intense absorption maximum located at 360 mμ. Concomitantly, no Dragendorff positive material could be detected on paper chromatograms. These changes are presumably associated with the formation of the third oxidative product, the identification and isolation of which are presently being studied in this laboratory.

DISCUSSION

The identification of 6-HPO as a product of nicotine degradation suggests that the bacterium P-34 metabolizes nicotine by a heretofore unreported pathway. Although 6-OHN had previously been found as a product of nicotine degradation (8), its further metabolism was reported to involve an attack upon the hydroxylated pyridine ring to yield a glutaconic acid derivative with an acid absorption maximum at 290 mμ which underwent a bathochromic shift to 300 mμ when the spectrum was determined in sodium hydroxide. This compound, although having absorption characteristics somewhat similar to those reported here for the product of 6-OHN oxidation, differs from 6-HPO in the following properties: the reported acid-base shift in absorption spectrum is not reversible (9); it reacts with ninhydrin; and analytical data indicate the presence of only one nitrogen in the molecule (8).

It is of interest to note that 6-hydroxymyosmine has been reported to be a product of the bacterial oxidation of nornicotine (10). This suggests that nornicotine is degraded in a manner similar to that reported for nicotine in this paper. However, since neither myosmine nor 6-hydroxynornicotine was detected, it is not clear whether nornicotine was initially hydroxylated and then dehydrogenated, or whether dehydrogenation preceded hydroxylation. Thus it is not possible to establish a formal analogy between the degradative pathways of nicotine and nornicotine, although the results are highly suggestive.

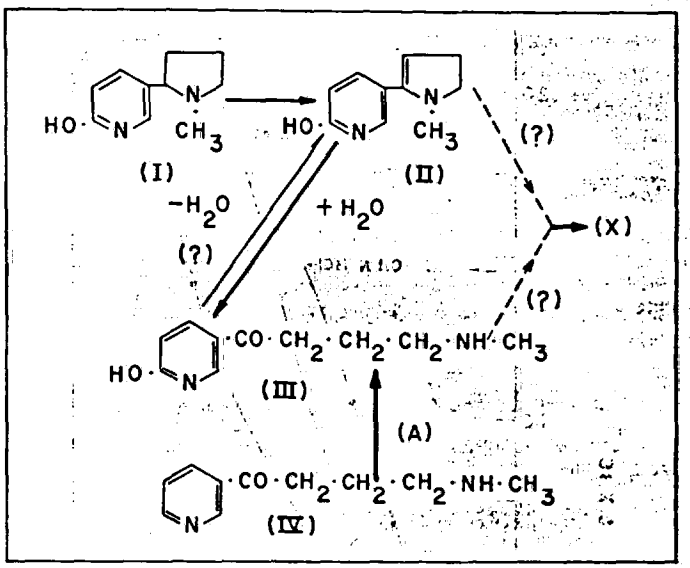

FIG. 6. Summary of reactions discussed. *I*, 6-hydroxynicotine; *II*, 6-hydroxy-*N*-methylmyosmine; *III*, 6-hydroxypseudooxynicotine; *IV*, pseudooxynicotine; *A*, hydroxylating enzyme; *X*, 3rd oxidative product of nicotine degradation.

The isolation of 6-HPO after the one-step oxidation of 6-OHN raises the question as to the nature of the immediate product of 6-OHN oxidation, since an examination of the structure of these compounds (Fig. 6) suggests that an intermediate exists between them. For reasons already mentioned, 6-hydroxy-*N*-methylmyosmine could be the intermediate. Its conversion to 6-HPO need not be enzymatic since as an α-pyrroline derivative it might be expected to be hydrolyzed spontaneously under physiological conditions to 6-HPO. Thus, an analogous compound, myosmine, was reported to undergo instantaneous hydrolysis in water to 3-pyridyl-ω-aminopropyl ketone (11). The reverse type of change, cyclization of the ketone pseudooxynicotine to methylmyosmine, occurs with alkalinization (12).

It is therefore not certain whether 6-HPO is a true intermediate in nicotine degradation or whether it is formed either because 6-hydroxy-*N*-methylmyosmine cannot be further oxidized in the reaction system devised for its accumulation or because 6-HPO is an artifact of the purification procedure used. Although possibly not stable in the free state under physiological conditions, 6-hydroxymethylmyosmine attached to an enzyme could be sufficiently stable to allow its further metabolism without the formation of 6-HPO. These possibilities are shown in Fig. 6. That 6-HPO is oxidized by resting cells and cell-free extracts in a manner consistent with its being an indermediate in nicotine degradation is not conclusive evidence on this point since one can postulate an equilibrium between the ketone and the myosmine derivative. It is hoped that identification of compounds further along the metabolic chain will ultimately allow a decision between these possibilities.

SUMMARY

An enzyme fraction has been obtained from a nicotine-oxidizing soil bacterium which catalyzes the aerobic oxidation of 6-hydroxynicotine with the consumption of 0.5 μmole of oxygen per μmole of substrate. The product isolated from this reaction has been identified as 6-hydroxypseudooxynicotine on the basis of elemental analysis, ultraviolet absorption spectra, and simi-

larity to the product of pseudooxynicotine hydroxylation by an enzyme fraction which is known to catalyze hydroxylation at the six position of the pyridine moiety.

Resting cells, crude extracts, and an ammonium sulfate fractionated extract of the nicotine-oxidizing organism oxidize 6-hydroxypseudooxynicotine. Under the appropriate conditions, oxidation by the fractionated extract ceases after the consumption of 0.5 μmole of oxygen per μmole of 6-hydroxypseudooxynicotine to yield a new ultraviolet absorbing substance which could be the third oxidative product of nicotine degradation.

The relation of 6-hydroxypseudooxynicotine to 6-hydroxy-*N*-methylmyosmine and the possible role of these compounds as intermediates in nicotine metabolism are discussed.

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